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Enclosed is a copy of Priority Document 01200353.9 EP filed Jan. 31, 2001, for the above referenced application.

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The attached documents are exact copies of the European patent application described on the following page, as originally filed.

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Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr. **Patent application No.** **Demande de brevet n°**

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The organization code and number of your priority application, to be used for filing abroad under the Paris Convention, is EP01200353

Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le President de l'Office européen des brevets
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Bezeichnung der Erfindung/Title of the invention/Titre de l'invention:
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Phosphorylatyed glyoxalase I and its use

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PHOSPHORYLATED GLYOXALASE I AND ITS USE

The present invention relates to a phosphorylated form of mammalian glyoxalase I. The present invention relates further to the use of phosphorylated mammalian glyoxalase I to modulate methylglyoxal (MG)-modification of proteins (AGE formation) and consequent cell death, especially upon stress such as oxidative stress, or upon TNF treatment.

Tumor Necrosis Factor (TNF) is a pleiotropic cytokine, originally described for its ability to cause hemorrhagic necrosis of certain tumors *in vivo* (Carswell et al., 1975). In addition to its anti-tumor and anti-malignant cell effects, TNF has been reported to influence mitogenesis, differentiation and immunoregulation of various cell types.
The activities of TNF are mediated through two cell-surface receptors, namely TNF-R55 (CD120a) and TNF-R75 (CD120b), which are expressed by most cell types. TNF's effects are mediated primarily through TNF-R55. Upon activation of the receptor, adaptor proteins such as TRADD and TRAF are recruited and bind to the intracellular part of the clustered receptor (for review, see Wallach et al., 1999). These receptor-associated molecules that initiate signaling events are largely specific to the TNF/nerve growth factor receptor family. However, the downstream signaling molecules are not unique to the TNF system, but also mediate effects of other inducers. Downstream signaling molecules in the TNF system identified so far include: caspases, phospholipases, the three mitogen-activated protein (MAP) kinases, and the NF-κB activation cascade.
TNF-induced cell death in L929 cells is characterized by a necrosis-like phenotype and does not involve DNA fragmentation (reviewed by Fiers et al., 1999). It is independent of caspase activation and cytochrome c release, but is dependent on mitochondria and is accompanied by increased production of reactive oxygen intermediates (ROI) in the mitochondria that are essential to the death process (Goossens et al., 1995; Goossens et al., 1999). The latter was demonstrated by the fact that lipophylic anti-oxidantia when added 3h after TNF treatment could not only arrest the ongoing increased ROI production but could also arrest cell death (Goossens et al., 1995). Furthermore, the mitochondria translocate from a dispersed distribution to a perinuclear cluster (De Vos et al., 2000); functional implications of this mitochondrial translocation remain unclear.

While much effort has been directed at the molecular mechanism of the caspase-dependent cell death pathway, relatively little is known about the TNF-induced ROI-dependent cell death pathway. To identify molecules involved in the latter, we performed a comparative study of the phosphoproteins from TNF-treated and control cells by two-dimensional (2-D) gel electrophoresis. It is known that upon activation of the TNF receptor, several kinases/phosphatases are activated (Guy et al., 1992; Guy et al., 1991). However, most of the changes in phosphorylation occur very rapidly (2-15 min) upon binding of TNF to its receptors and most of them are transient and related to the gene-inductive activities of TNF.

To identify molecules that are involved in the cytotoxic process downstream of the receptor-proximal events, lysates from cells that had been stimulated with TNF for 1.5h are studied. Previously, oncoprotein 18 (Op18, stathmin) has been identified as a protein with reproducible and large increases in phosphorylation upon TNF-treatment. Op18 is responsible for TNF-induced microtubule stabilization that promotes cell death (Vancompernolle et al., 2000). Unexpectedly, we were able to demonstrate that glyoxalase I is also phosphorylated upon TNF treatment. Phosphorylated mammalian glyoxalase I has not yet been described.

Glyoxalase I, together with glyoxalase II, constitutes the glyoxalase system that is an integral component of the cellular metabolism of α-ketoaldehydes and is responsible for the detoxification of the latter. The prime physiological substrate of the glyoxalase system is methylglyoxal (MG), which is cytotoxic. The major source of intracellular MG is the glycolysis namely, non-enzymatic and enzymatic elimination of phosphate from dihydroxyacetone phosphate and glyceraldehyde-3-phosphate. The glyoxalase system, using glutathione (GSH) as cofactor, catalyzes the conversion of methylglyoxal to D-lactate in two consecutive steps (Racker, 1951). Glyoxalase I catalyses the isomerization of the hemithioacetal, produced by the nonenzymatic conjugation of methylglyoxal with glutathione (GSH), to S-D-lactoylglutathione which is then hydrolysed by glyoxalase II to D-lactate and GSH. D-lactate is then further metabolized to pyruvate by 2-hydroxy-acid dehydrogenase localized in the mitochondria. In addition to its role as detoxification system, it has been suggested that glyoxalase I, together with its substrate

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MG, is involved in the regulation of cellular growth (for a review see Kalapos, 1999), but until now this role has not been found. Increased expression of glyoxalase I occurs in diabetic patients and in some types of tumor such as colon carcinoma (Ranganathan et al., 1993) and prostate cancer (Davidson et al., 1999). Also hypoxia can lead to increased expression of glyoxalase I (Principato et al., 1990). Recently, it has been shown that glyoxalase I is involved in resistance of human leukemia cells to antitumor agent-induced apoptosis (Sakamoto et al., 2000).

It is a first aspect of the invention to provide phosphorylated mammalian glyoxalase. The phosphorylation may be a single or a multiple phosphorylation. A preferred embodiment is a phosphorylated mammalian glyoxalase I comprising SEQ ID N° 1. Preferably, phosphorylation is carried out at position Ser 8 and/or Ser 21 and/or Ser 26 and/or Thr 107.

Another aspect of the invention is the use of a phosphorylated glyoxalase I to modulate MG-modification of proteins. Said phosphorylated glyoxalase I may be any glyoxalase I, know to the person skilled in the art, such as a fungal glyoxalase I or a plant glyoxalase I. Preferably, said glyoxalase I is a mammalian glyoxalase I. MG-modified proteins or advanced glycation end products (AGEs) are known to be synthezised in response upon a number of pathophysiological conditions *in vivo*, such as cataract formation (Shamsi 2000), vascular complications associated with chronic diabetes (Shinohara et al., 1998), tissue damage after ischemia/reperfusion (Oya et al., 1999) and aging (Corman et al., 1998). The term AGE, as used here, is used for any MG-modification of a protein, irrespective the way it is formed; the term MG-modification of proteins is considered as being equivalent with the term AGE formation.

Still another aspect of the invention is the use of phosphorylated glyoxalase I, or an inhibitor of the phosphorylation of glyoxalase I, preferably mammalian glyoxalase I to modulated TNF induced cell death. Said inhibitor can be any inhibitor that inhibit the phosphorylation of glyoxalase I; preferably said inhibitor is an inhibitor of the PKA activity.

A further aspect of the invention is the use of phosphorylated glyoxalase I, or an inhibitor of the phosphorylation of glyoxalase I to modulate stress induced cell death. Preferably, said stress is oxidative stress. Oxidative stress, followed by ROI induction and AGE

formation is known to occur in several organisms, including plants, yeast, fungi and mammalians. A preferred embodiment is the use of mammalian phosphorylated glyoxalase I to modulate oxidative stress induced cell death.

Still another aspect of the invention is the use of PKA to phosphorylate glyoxalase I. By modulating the phosphorylation of glyoxalase I, TNF induced cell death and stress induced cell death, preferably oxidative stress, can be modulated.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: phosphorylation of glyoxalase I in control cells (left panel) and after 1.5 h of TNF treatment (right panel).

Figure 2: effect of the glyoxalase I inhibitor S-p-bromobenzylglutathione diester on TNF-induced cytotoxicity in L929s cells, in function of the incubation time. TNF is added at a concentration of 1000 units/ml; the inhibitor is added 1h10min prior to TNF at a concentration of 10 µM (GI10) or 20 µM (GI20). The time scale is calculated from the moment of TNF addition

Figure 3: western blots, developed with anti-human glyoxalase I polyclonal antibody, of 2-dimensional gels (pH 3-10) from total cell lysates derived from control cells (C), glyoxalase I inhibitor S-p-bromobenzylglutathione diester (20 µM)-treated cells (I), TNF treated cells (TNF) and cells treated with TNF and glyoxalase I inhibitor (TNF + I), cells treated with the PKA inhibitor H89 (5 µM) (H89) and cells treated with TNF and H89 (TNF + H89).

Figure 4: effect of different concentrations exogeneously added methylglyoxal on TNF-induced cell death. Measurement after 5,5 h of incubation with TNF and methylglyoxal at a concentration as indicated.

Figure 5: effect of different concentrations of the AGE formation inhibitor, aminoguanidine, on the TNF-induced cell death. Measurement after 16h of incubation with TNF and aminoguanidine at a concentration as indicated.

Figure 6: inhibition of TNF induced cell death by the PKA inhibitor H89 (2 h pre-treatment), in function of the incubation time. The concentration of the inhibitor H89 is as indicated on the graph. The time scale is calculated from the moment of the TNF addition.

EXAMPLES

Materials and methods to the examples

Cell lines and cultures

All L929 cells were cultured in Dulbecco's modified Eagle's medium supplemented with heat-inactivated fetal calf serum (5% v/v), heat-inactivated newborn calf serum (5% v/v), penicillin (100 units/ml), streptomycin (0.1 mg/ml), and L-glutamine (2mM), at 37°C in a humidified incubator under a 5% CO_2 atmosphere.

Reagents

Murine TNF (mTNF) was produced in E. coli and purified to at least 99% homogeneity in our laboratory. It had a specific activity of 1.9×10^8IU/mg of protein (National Institute for Biological Standards and Control, Potters Bar, UK), contained 4ng of endotoxin/mg protein, and was used at 1000IU/ml. Propidium iodide (PI) and cycloheximide (CHX) (all from Sigma) were used at concentrations of 30µM and 50µg/ml, respectively.

Measurement of TNF-induced cell death by flow cytometry

Cell death in L929 was induced by addition of TNF to the cell suspension. Cell death was measured by quantifying PI-positive cells by FACS (FACSCalibur, Becton Dickinson, San Jose, CA). The PI dye was excited with an argon-ion laser at 488nm; PI fluorescence was measured above 590nm using a long-pass filter. Routinely, 3,000 cells were analyzed. Cell death is expressed as the percentage of PI-positive cells in the total cell population.

Radiolabeling of cells and preparation of the sub-cellular protein fractions

L929 cells were plated 48h prior to the experiment. ^{32}P labeling was carried out as described in (Guy et al., 1992). TNF treatments (1000 IU/ml, 1.5h) were done in the presence of cycloheximide (CHX), to synchronize cell death. To simplify the 2-D phosphoprotein pattern and subsequent computer-assisted analysis, we prepared two subcellular protein fractions. The cytosolic protein fraction, containing soluble cytoplasmic molecules and molecules derived from single-membrane organelles, was

obtained as the supernatant from digitonin (0.03%)-permeabilised cells. After rinsing once with excess PBS buffer, the remaining cell fraction was lysed in a CHAPS (2%)-containing buffer as described in (Guy et al., 1992). This lysate was then centrifuged (20,000g) and the supernatant was used as the organelle fraction; it is enriched for mitochondrial and cytoskeleton-derived proteins.

Two-dimensional (2D) gel electrophoresis

Isoelectric focusing. Isoelectric focusing was carried out on 18 cm IPG strips, pH 4-7 or pH 3-10 (Amersham Pharmacia Biotech), according to the manufacturer's instructions. Protein samples were precipitated with ethanol and redissolved in lysis buffer.

SDS-PAGE. The second dimension (SDS-PAGE) was run on large vertical gels (12.5 % acrylamide, Biorad).

Western blotting

Proteins were separated by SDS-PAGE (12.5 %) and transferred to a PVDF membrane (Hybond-P, Amersham Pharmacia Biotech). The blots were incubated with an anti-human glyoxalase I antibody, followed by ECL-based detection (Amersham Pharmacia Biotech).

Amino acid sequence analysis by MALDI-mass spectrometry

Following in-gel digestion of the excised protein with endoproteinase Lys-C (sequencing grade; Boehringer, Mannheim, Germany), a 10% aliquot of the generated peptide mixture was purified and concentrated on Poros® 50 R2 beads (Gevaert et al., 1998; Gevaert et al., 1997) and used for MALDI-MS peptide mass fingerprint analysis. However, partly due to contamination with human keratin peptides, the obtained peptide mass map did not lead to any unambiguous protein identification in a non-redundant protein database. Therefore the remainder of the peptide mixture was separated by RP-HPLC, a total of 20 fractions containing eluting peptides were obtained, which were all analysed by MALDI-MS. Adequate peptide ions were further selected for post-source decay (PSD) analysis (Spengler et al., 1992). A PSD-spectrum obtained from a peptide ion with a mass of 902.42 Da (measured in linear mode) present in the first RP-HPLC

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fraction, could be unambiguously assigned to the peptide NH_2-SLDFYTR-COOH present in human glyoxalase I (database entry number 417246) using the SEQUEST algorithm and a non-redundant protein database. Following a search in an EST-database the same peptide sequence was identified in many different mouse EST-clones. The identified peptide contains an arginine residue at its C-terminus instead of a lysine; an observation which we made several times when endoproteinase Lys-C was used as the protease.

In order to confirm our initial finding, PSD-analysis was conducted on a peptide with an apparent mass of 1396.53 Da present in RP-HPLC fraction 11. Based on the partially ^{18}O-labelled y-type fragment ions, a peptide sequence tag (391.24)YAI/LF(885.67) could easily be obtained. Furthermore a SEQUEST-search in a non-redundant protein database lead to the identification of the peptide NH_2-FSLYFLAYEDK-COOH also belonging to human glyoxalase I. Again the same peptide sequence was found in different mouse EST-clones using the PSD-data and a SEQUEST-search in an EST-database. Based upon the amino acid sequence of human glyoxalase I, masses of peptide ions observed in the different RP-HPLC fractions could be assigned to the identified protein. Hereby, a total of 38% of the amino acid sequence of the protein was covered, again confirming the identification of glyoxalase I.

Assay of glyoxalase I activity

The glyoxalase I assay was performed according to a spectrophotometric method monitoring the increase in absorbance at 240 nm due to the formation of S-D-lactoylglutathione for 4 min at 20°C. The standard assay mixture contained 2 mM MG and 2 mM GSH in a sodium phosphate buffer (50 mM, pH 6.6). Before initiating the reaction by adding the total cytosolic protein fraction to the assay mixture, the mixture was allowed to stand for 10 min to ensure the equilibration of hemithioacetal formation.

D-Lactate measurements

D-Lactate measurements were performed by a fluorimetric assay using an endpoint enzymatic assay with D-lactate dehydrogenase (McLellan et al. 1992).

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Intracellular methylglyoxal measurements

Intracellular free methylglyoxal is detected as the 2-methylquinoxaline (2-MQ) derivative of methylglyoxal formed with o-phenylenediamine (o-PD) using the general approach of (Chaplen et al., 1996). Samples arrived frozen on dry ice and were stored at 20 °C until assayed. Samples were thawed at room temperature and maintained on ice during the assay procedure. Sample volume was increased to 2.5 ml with MilliQ water and the samples were sonicated (5 s, 30 W). 5 M $HClO_4$ (PCA; 0.25 ml) was added to precipitate macromolecules and the resulting mixture was incubated on ice for 20 min. Samples were then centrifuged (12,000xg, 10 min) to remove precipitated materials. The supernatant was passed through a C-18 SPE cartridge (Waters Sep-Pak tC18 plus cartridge, Millipore Corp, Marlborough, MA) that had been prepared by flushing with 6-8 ml of acetonitrile followed by 6-8 ml of 10 mM KH_2PO_4 (pH 2.5, adjusted with concentrated H_3PO_4). The pre-derivatization SPE step removes phenol red and other interfering compounds. Samples were supplemented with 12.5 nmol 5-methylquinoxaline (5-MQ; internal standard) and 250 nmol o-PD (derivatizing agent) and reacted at 20 °C for 3.5 to 4 h.

SampleConcentration

All samples are concentrated after derivatization. For concentration, the samples are passed through a C-18 SPE cartridge, prepared as described above, at a rate of 1-2 ml/min. The cartridges are then rinsed with 1-2 ml 10 mM KH_2PO_4 (pH 2.5) and the retentate eluted with 2 ml if acetonitrile. Eluates were evaporated to a volume of 200 ml using a Savant Speed-Vac Concentrator vacuum centrifugation unit (Savant Instruments, Farmingdate, NY) and filtered through 0.2-mm Gelman PVDF filters (Fisher Scientific, Chicago, IL) into sample vials.

HPLC of Quinoxalines

HPLC was performed as described previously (Chaplen et al., 1996) but with a mobile phase consisting of 35% acetonitrile/0.1% trifluoroacetic acid, pH 2.4 and 65% 10 mM phosphate/0.1% trifluoroacetic acetic acid in HPLC grade water, pH 2.4. Under these modified conditions, 2-MQ eluted after 7.5 min and 5-MQ eluted after 11.2 min.

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Example 1: TNF induces increased phosphorylation of Glyoxalase I

Fig. 1 shows the autoradiogram of the two-dimensional gels from TNF-treated and control samples that were derived from cells labeled with ^{32}P-orthophosphate. The protein spot with increased phosphorylation identified as glyoxalase I is indicated by an arrow. It was identified by mass spectrometry analysis of a peptide mixture derived from an in-gel digestion of the excised protein spot. The increased phosphorylation of glyoxalase I is already observed after 15 min of TNF treatment, but is much more pronounced after 1.5h of TNF treatment (Fig. 1). This indicates that the TNF-induced phosphorylation of GLO1 is an early but lasting event. Phosphorylation of mammalian glyoxalase I has not yet been described, but the sequence does contain several potential phosphorylation sites (Ranganathan et al., 1993). Interestingly, phosphorylation of yeast GLO1 has been observed during the sexual response of *S. cerevisiae* – specifically, during the arrest of cell division at the G1 phase, which occurs when haploid cells of one sex are exposed to the mating factor of the opposite type of cells (Inoue et al., 1990).

Example 2: The Glyoxalase I inhibitor S-p-bromobenzylglutathione diethylester inhibits TNF-induced cell death

To examine the role of glyoxalase I in TNF-induced cell death, we tested the effect of the cell permeable competitive inhibitor of glyoxalase I S-p-bromobenzylglutathione diester on cell death. Preincubation (1h10min) of L929 cells with this inhibitor strongly inhibits TNF-induced cell death in a concentration dependent manner (Fig. 2). An inhibition of 60% was obtained at a concentration of 20 µM of the inhibitor. However, when the inhibitor and TNF were added at the same time, a synergistic effect on TNF-induced cell death was obtained (50% increase in cell death at a concentration of 20 µM of the inhibitor). This synergistic effect is more pronounced at lower doses of TNF; that is, when the cells die more slowly.

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Example 3: The Glyoxalase I inhibitor S-p-bromobenzylglutathione diethylester inhibits the TNF-induced phoshorylation of glyoxalase I

This differential effect of the inhibitor on TNF-induced cell death prompted us to investigate whether the binding of the inhibitor to GLO1 competes and thus inhibits the TNF-induced phosphorylation of GLO1. Fig.3 shows the Western blots, developed with an anti-human glyoxalase I polyclonal antibody, of 2-dimensional gels (pH 3-10) from total cell lysates derived from TNF-treated and control cells and from TNF-treated and control cells that were first preincubated with the glyoxalase I inhibitor for 1h10min. In the upper panels, one can see that TNF induces a more acidic phosphoisoform of glyoxalase I which is not well separated from the non-phosphorylated form (fills the space between the most left isoform and the main non-phosphorylated form. In the lower panels, one can see that in the presence of the glyoxalase I inhibitor TNF cannot induce the more acidic phosphoisoform of glyoxalase I (identical 2-D patterns as in the control). These data show that the competitive inhibitor of glyoxalase I S-p-bromobenzylglutathione inhibits the TNF-induced phosphorylation of GLO1 and that phosphorylated glyoxalase I is essential for cell death. These data also suggest that phosphorylation of glyoxalase I modulates the active site of the enzyme.

Thus, the differential effect of the GLO1 inhibitor on TNF-induced cell death can be explained as follows:

- when the cells are pre-treated with the inhibitor, the inhibitor is already bound to GLO1 and thus hinders the TNF-induced phosphorylation of GLO1 which then leads to inhibition of phosphorylated GLO1-mediated MG-modification of proteins and consequent cell death.
- however, when the cells are treated with the inhibitor and TNF together, the TNF-induced phosphorylation of GLO1 occurs first (via a receptor-activated kinase cascade) and the inhibitor can only bind to non-phosphorylated GLO1 (and not to phosphorylated GLO1) leading to inhibition of GLO1 and thus to accumulation of MG, resulting in phosphorylated GLO1-mediated MG-modification of proteins and consequent cell death.

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Example 4: TNF-induced phosphorylation of glyoxalse I does not inhibit methylglyoxal detoxification

For many years, α-ketoaldehydes, exemplified by methylglyoxal, have been known to be carcinostatic, but their direct use as anti-cancer drugs is prevented by their rapid detoxification *in vivo* by the glyoxalase system. Therefore, glyoxalase I inhibitors have been developed as potential anti-cancer agents (Vince and Wadd, 1969) (Thornalley et al., 1996). Bearing this in mind, one would expect that TNF-induced phosphorylation of GLO1 would result in inhibition of the enzyme and thus in accumulation of MG with cytotoxicity as a consequence. However, our experiments with the GLO1 inhibitor do not support this expectation, because we would then expect a synergistic effect of the pre-incubated inhibitor on TNF-induced cell death. Indeed, measurements of GLO1 activity in lysates derived from TNF-treated and control cells showed no inhibition, but even a limited increase in GLO1 activity in TNF-treated cells. These experiments were repeated several times and each time gave the same results, with an average increase of 8% after 1h of TNF-treatment and 12% (from 0,086 ±0,003 to 0,106±0,001 units per 8,5µg of total protein) after 1.5h of TNF-treatment. Measurement of the concentration of the end product of the glyoxalase system D-lactate showed an increase of 60% after 1.5h of TNF-treatment compared to control cells. This further confirmed that TNF did not inhibit GLO1 activity and that an increased flux of MG is converted through the glyoxalase system in TNF-treated cells.

Example 5: TNF increases the intracellular concentrations of methylglyoxal

As we consider it very unlikely that TNF would cause an increased detoxification of MG through the glyoxalase system, a more plausible explanation is that TNF induces an increase in the intracellular concentrations of MG via a pathway other than inhibition of glyoxalase I. An increase in the intracellular concentration of MG would then also automatically result in an increased flux of MG through the glyoxalase system and an increased GLO1 activity. Therefore, intracellular concentrations of MG were measured with a method that not only measures free MG, but also MG bound to biological molecules (majority of the MG), mainly proteins (Chaplen et al., 1998). Two independent

experiments were performed in which intracellular concentrations of MG were measured in TNF-treated (1,5h) L929 cells compared to control cells. Each sample was measured in triplicate and each time gave very reproducible results. These results showed that TNF strongly increased the intracellular concentrations of MG, with an increase of 32% (from 0,91µMole in control cells to 1,20µMole in TNF-treated cells) in the one experiment and 94% (from 1,24µMole in control cells to 2,39µMole in TNF-treated cells) in the other experiment.

Also, exogeneously added MG is strongly synergistic with TNF-induced cell death in a concentration dependent manner (Fig 4), while MG alone and used at the same concentrations is not cytotoxic for L929 cells. The synergistic effect of exogeneously added MG is more pronounced at lower doses of TNF (100 U/ml) and also earlier in TNF treatment. This result can be explained by the fact that the TNF-induced increase of endogeneous MG is more drastic at higher doses of TNF (1000U/ml) and later in TNF treatment.

Example 6: Inhibition of AGE formation inhibits TNF-induced cell death

Increased endogenously produced levels of dicarbonyls, especially methylglyoxal, are involved in numerous pathogenic processes *in vivo*, including the formation of advanced glycation end-products (AGEs) which contribute to the pathophysiology of aging and to complications associated with chronic diabetes. They have been detected in several pathophysiological conditions *in vivo*, such as cataract formation, vascular complications in diabetes, and tissue damage after ischemia/reperfusion. All these conditions are characterized by increased oxidative stress, and recently it was shown that mitochondrial ROS are the direct cause of increased concentrations of MG and thus AGEs formation in diabetic hyperglycaemia (Nishikawa et al., 2000). Since TNF-induced cell death in L929 cells is characterized by increased production of mitochondrial ROS (Goossens et al., 1995) (Goossens et al., 1999) which are essential for cell death and increased levels of MG, we tested whether irreversible protein modification by MG plays a role in TNF-induced cell death. For this we used aminoguanidine, a nucleophilic hydrazine compound and inhibitor of AGE formation *in vivo* (Brownlee et al., 1986). The

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percentage of cell death in L929 cells after 16h of TNF treatment (20 U/ml) with and without aminoguanidine is shown in Fig. 5. A maximum inhibition of cell death of 25% was obtained in the presence of 600 or 800 µM of aminoguanidine and 15% inhibition in the presence of 400µM. This inhibition was less pronounced (average of 15% to 20%) when the cells died more rapidly by giving higher doses of TNF (500-1000U/ml). This could be due to the fact that the reaction of aminoguanidine with MG and MG-modified proteins is rather slow and that the MG protein modifications that occur during TNF-induced cell death are more rapid at higher doses of TNF and could even be enzymatically catalysed. These data indicate that irreversible protein modification by methylglyoxal might play a role in TNF-induced cell death.

Example 7: The PKA inhibitor inhibits TNF-induced cell death and TNF-induced phosphorylation of glyoxalase I

As it has been already shown that PKA is activated by TNF (Zhang et al., 1988), we examined whether pretreatment (2h) of L929 cells with the PKA inhibitor H89 had an effect on TNF-induced cell death. As shown in Fig. 6, the PKA inhibitor inhibits TNF-induced cell death in a concentration-dependent fashion and to a similar extent as the glyoxalase I inhibitor. Even an inhibitory effect was already obtained at relatively low concentrations of the inhibitor (1 µM), while at the highest concentration (5 µM) an inhibition of more then 50% was obtained. These data thus indicate that PKA plays a role in TNF-induced cell death. Next, we examined whether PKA was also responsible for the TNF-induced phosphoryaltion of endogeneous glyoxalase I in L929 cells. As shown in Fig. 3, pretreatment of the cells with the PKA inhibitor completely abolished the induction of the more acidic isoform by TNF. This suggests that the inhibitory effect of the PKA inhibitor on TNF-induced cell death could be largely due to the inhibition of phosphorylation of glyoxalase

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SEQUENCE LISTING

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35 40 45

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Ile Pro Lys Glu Lys Asp Glu Lys Ile Ala Trp Ala Leu Ser Arg Lys
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Pro Asn Lys Met Ala Thr Leu Met
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KVC/Glyox/079

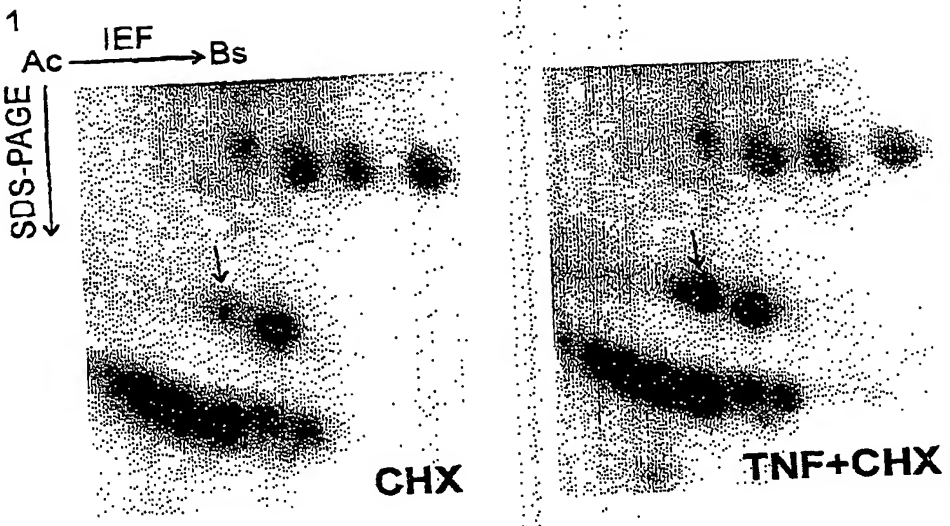
CLAIMS

1. A phosphorylated mammalian glyoxalase I.
2. The phosphorylated mammalian glyoxalase I according to claim 1, comprising SEQ ID N°1.
3. The use of a phosphorylated glyoxalase I and/or an inhibitor of the phosphorylation to modulate MG-modification of proteins.
4. The use of a phosphorylated glyoxalase I and/or an inhibitor of the phosphorylation to modulate TNF induced cell death.
5. The use of a phosphorylated glyoxalase I and/or an inhibitor of the phosphorylation to modulate cell death.
6. The use of a phosphorylated glyoxalase I and/or an inhibitor of the phosphorylation according to claim 5, whereby said stress is oxidative stress.
7. The use of a phosphorylated glyoxalase I and/or an inhibitor of the phosphorylation according to claim 3-6, whereby said phosphorylated glyoxalase I is a mammalian glyoxalase I.
8. The use of a phosphorylated glyoxalase I and/or an inhibitor of the phosphorylation according to claim 3-6, whereby said inhibitor is a PKA inhibitor.
9. The use of PKA to phosphorylate glyoxalase I.

ABSTRACT

The present invention relates to a phosphorylated form of mammalian glyoxalase I. The present invention relates further to the use of phosphorylated mammalian glyoxalase I to modulate MG-modification of proteins (AGE formation) and consequent cell death, especially upon stress such as oxidative stress, or upon TNF treatment.

Fig. 1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IEF
Bs
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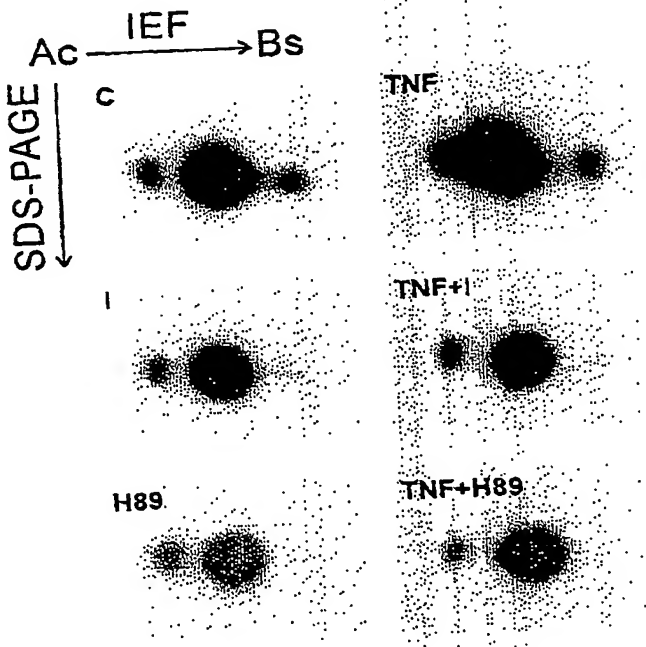
CHX
TNF+CHX

Fig. 3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TNF
I
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TNF+H89

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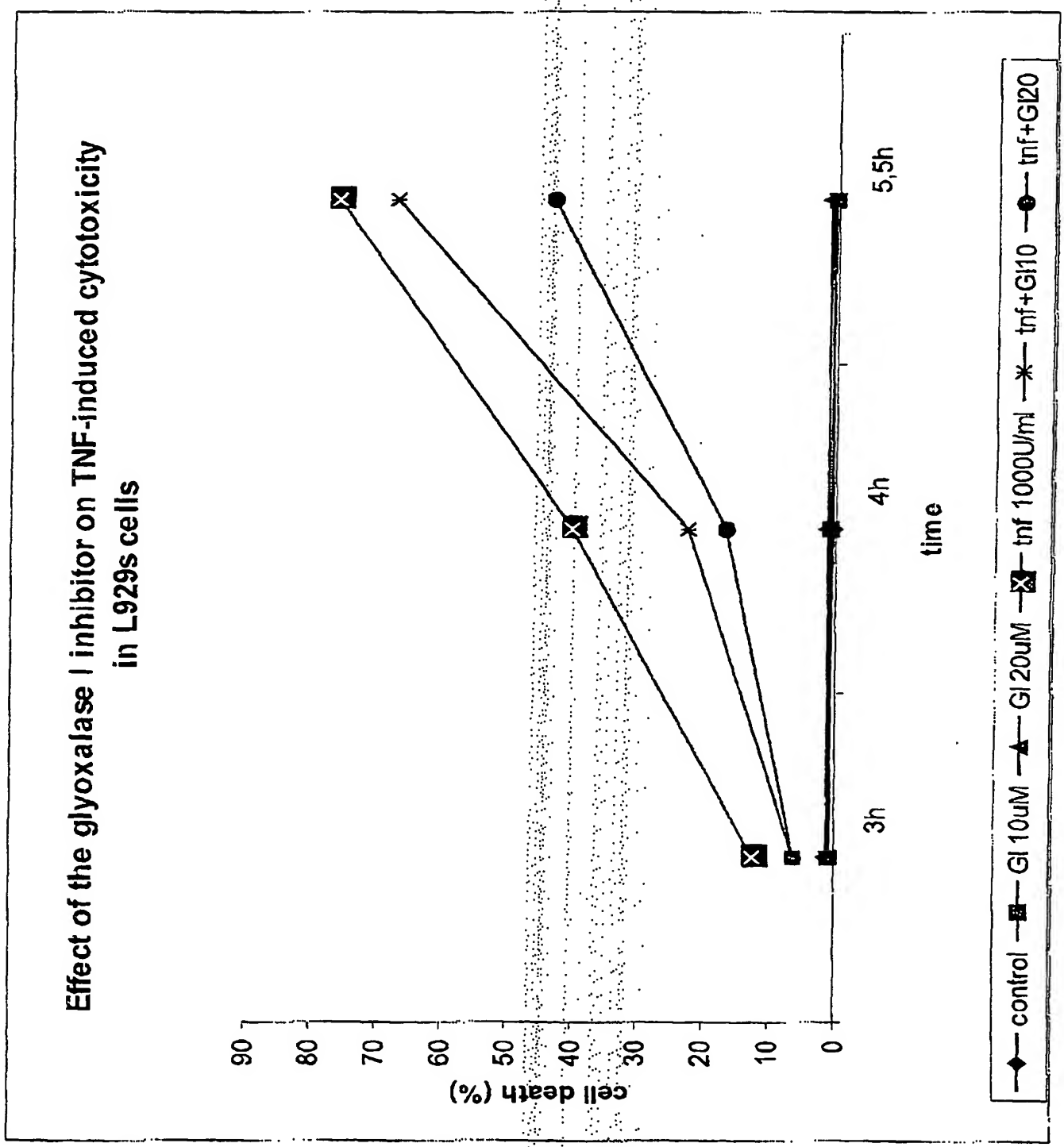

Effect of the glyoxalase I inhibitor on TNF-induced cytotoxicity in L929s cells
cell death (%)
90
80
70
60
50
40
30
20
10
0
3h
4h
5,5h
time
control
GI 10uM
GI 20uM
tnf 1000U/ml
tnf+GI10
tnf+GI20

Fig. 4

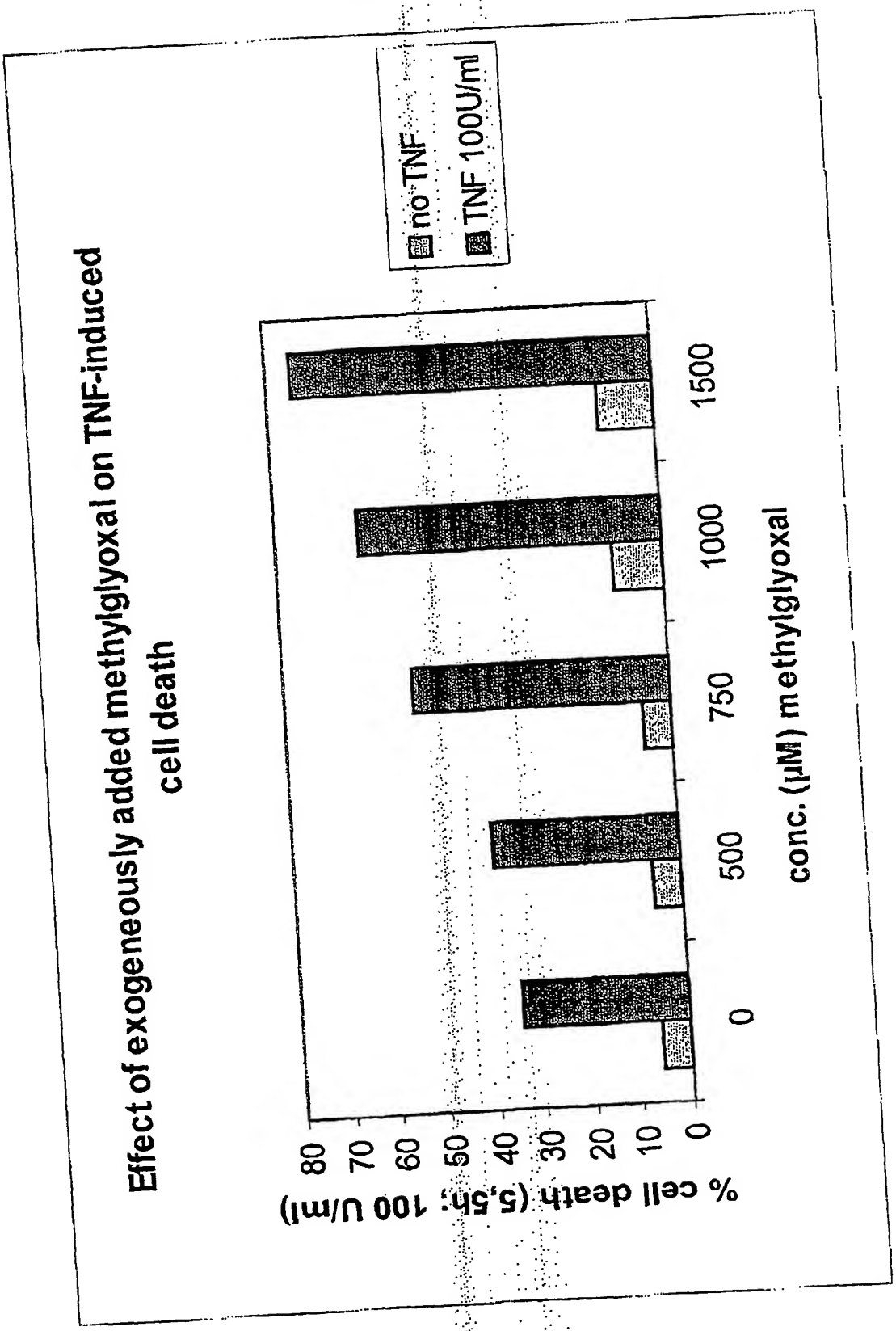

Effect of exogeneously added methylglyoxal on TNF-induced cell death
no TNF
TNF 100U/ml
% cell death (5,5h; 100 U/ml)
0
10
20
30
40
50
60
70
80
conc. (µM) methylglyoxal
0
500
750
1000
1500

Fig. 5

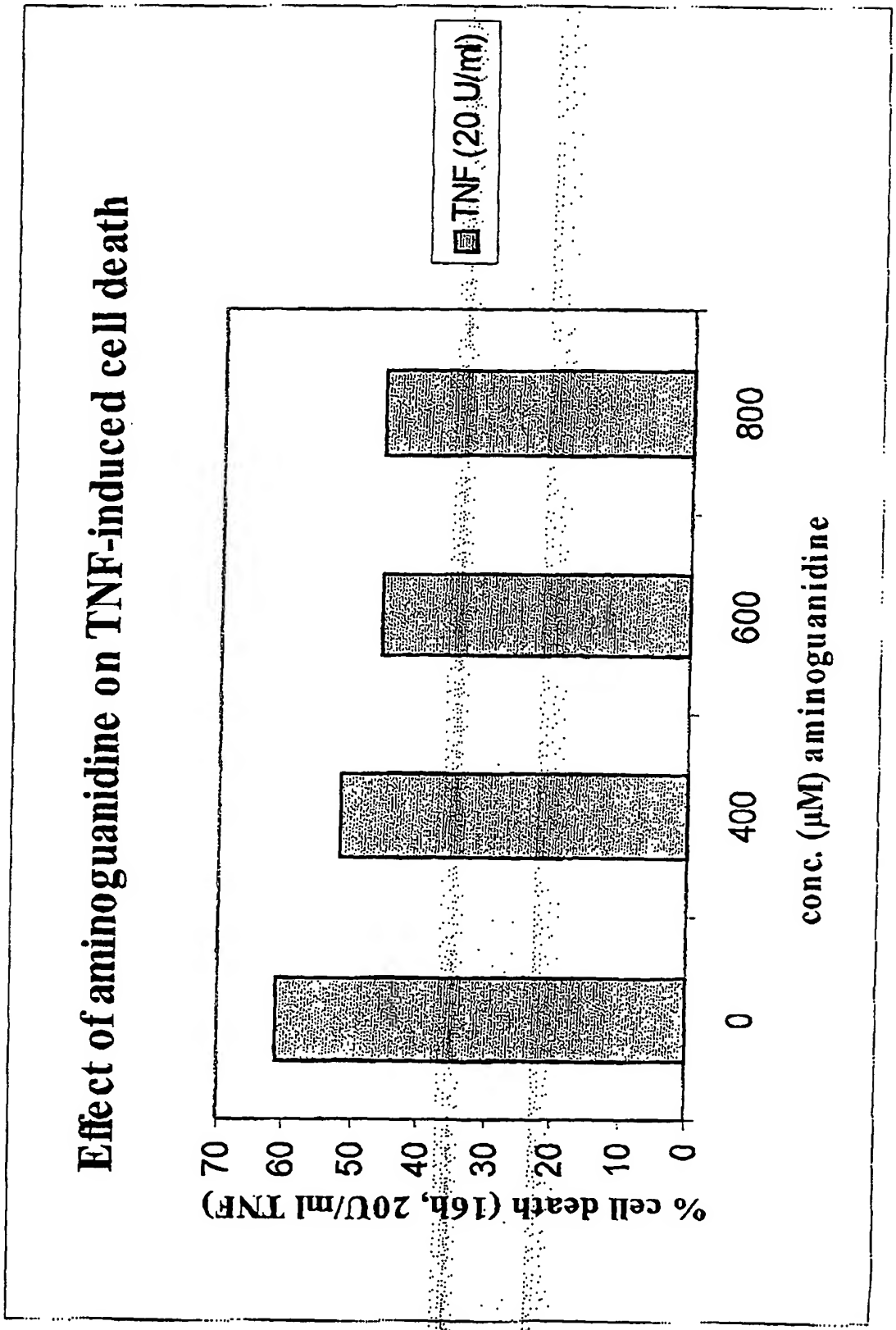
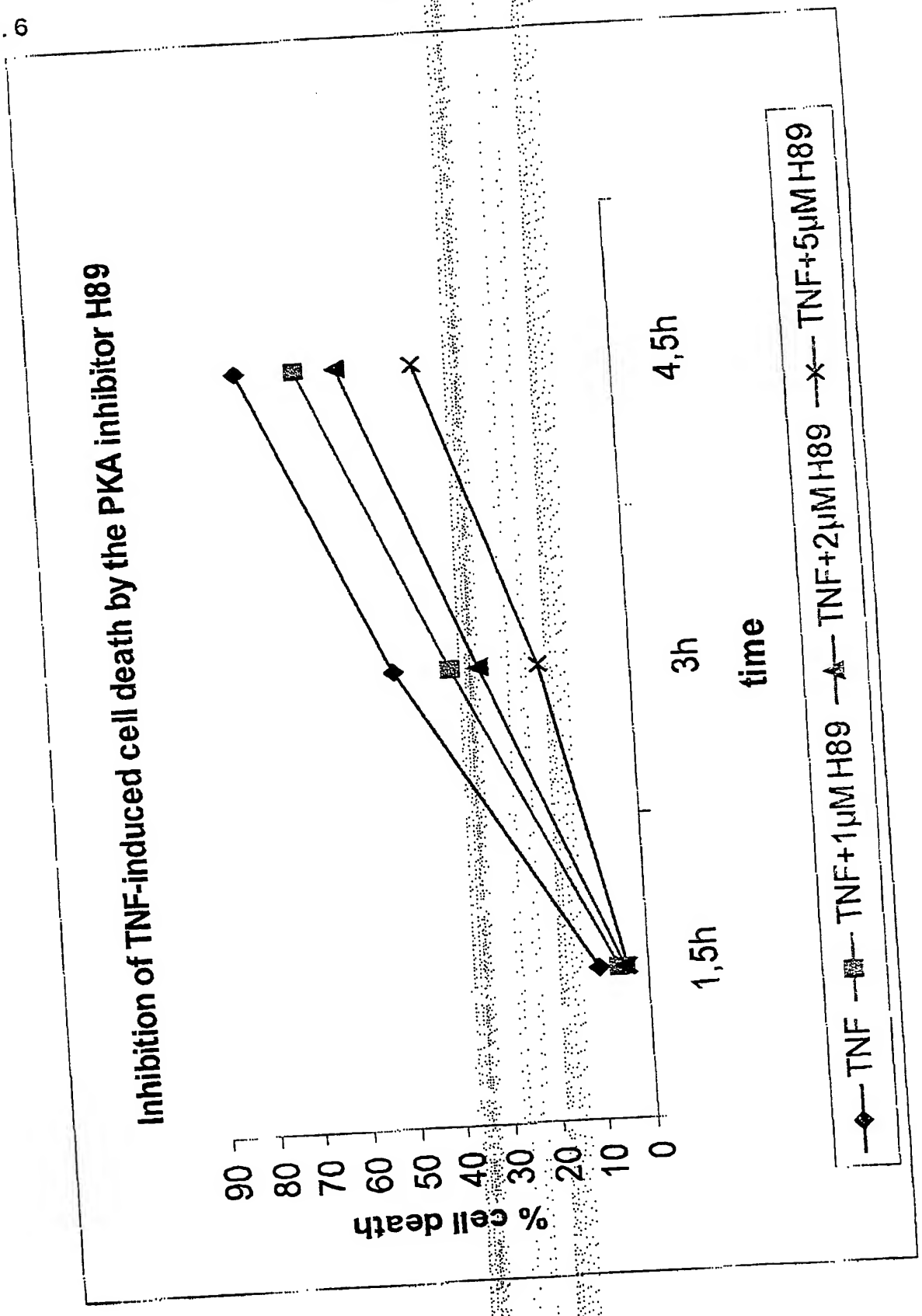

Effect of aminoguanidine on TNF-induced cell death
TNF (20 U/ml)
% cell death (16h, 20U/ml TNF)
0
10
20
30
40
50
60
70
conc. (µM) aminoguanidine
0
400
600
800

Fig. 6


Inhibition of TNF-induced cell death by the PKA inhibitor H89
% cell death
0
10
20
30
40
50
60
70
80
90
time
1,5h
3h
4,5h
TNF
TNF+1µM H89
TNF+2µM H89
TNF+5µM H89